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The effect of starvation on the control of phosphofructokinase activity in the epithelial cells of the rat small intestine

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1. The effect of depriving rats of food for 48 h on the specific activity of phosphofructokinase in the epithelial cells of the small intestine and on the regulatory properties of the enzyme displayed in crude (particle-free) mucosal extracts was studied. 2. The specific activity of phosphofructokinase, measured under optimal conditions at pH 8, in the mucosa of fed rats showed a negative aboral gradient along the intestine, decreasing from 15.2 ± 1.2 units (μmol/min)/g wet wt. in the proximal jejunum to 4.6 ± 1.2 units/g wet wt. in the terminal ileum. 3. After starvation, the gradient was diminished, but not abolished; the diminution in gradient was due almost exclusively to a decrease in the specific activity of phosphofructokinase in the proximal jejunum by about 30%, there being no change in the terminal ileum. 4. In fed rats, the susceptibility of phosphofructokinase to inhibition by ATP, when assayed in crude mucosal extracts under suboptimal conditions, was independent of length along the small intestine; the ratio of the activity observed at pH 7.0 in the presence of 0.5 mM-fructose 6-phosphate and 2.5 mM-ATP to the optimal activity at pH 8, $v_{0.5}/V$, was 0.36 ± 0.05 in the proximal jejunum and 0.42 ± 0.07 in the terminal ileum. 5. After starvation, the susceptibility of phosphofructokinase to inhibition by ATP was increased and was again found to be independent of length along the small intestine: after starvation, $v_{0.5}/V$ was 0.19 ± 0.04 and 0.20 ± 0.07 for the proximal jejunum and the terminal ileum respectively. 6. Re-feeding of previously starved rats on a high-carbohydrate diet overnight for 16 h restored both the specific activities of phosphofructokinase and its susceptibility to inhibition by ATP to normal values for fed rats. 7. The data support the idea that the specific activities and the regulatory properties of phosphofructokinase in the epithelial cells of rat small intestine are mediated by distinct humoral factors. 8. The changes in glucose utilization rate of the jejunum when rats are starved can in principle be accounted for by a combination of changes in the specific activity and in the regulatory properties of mucosal phosphofructokinase.

It is well established that the rate of glycolysis in rat small intestine decreases significantly on depriving the animal of food. For example, in studies with a particle-free supernatant derived from homogenates of the mucosa of whole small intestine, Srivastava & Hübscher (1966) found that the rate of lactate production in 48 h-starved rats was lowered to about 20% of that observed for fed rats (see also Anderson, 1974). Similar decreases have also been observed in intact tissue. Thus, in studies with a vascularly-perfused preparation of jejunum, Hanson & Parsons (1978) found a lowering of the rate of lactate production after a 48 h starvation period to about 52% of that for the fed rat, and a corresponding decrease in the rate of glucose utilization to about 36%; the difference in the changes of the rates of lactate production and glucose utilization was ascribed to changes in the rate of glucose oxidation.

The rate-limiting enzyme of glycolysis in rat intestinal epithelial cells is thought to depend on the nature of the substrate, whether glucose or glucose 6-phosphate. With the latter, there appears to be general agreement that phosphofructokinase is rate-limiting (Srivastava & Hübscher, 1966; Shoaf *et al.*, 1981). However, with glucose as substrate, hexo-

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kinase appears to be rate-limiting (Srivastava & Hübscher, 1966), a finding in keeping with the observation that simple preparations of rat small intestine studied *in vitro*, e.g. rings, slices, everted sacs and loops perfused in non-segmented flow, do not show a Pasteur effect. The lack of a Pasteur effect has been attributed by Ramaiah and co-workers to a favourable ratio of activators to inhibitors, so that the activity of phosphofructokinase is unrestrained (Tejwani & Ramaiah, 1971; Tejwani *et al.*, 1974; Ramaiah, 1974); nevertheless, the possibility should be noted that such a favourable ratio could be an artifact arising from lack of adequate oxygenation in these simple preparations, for a Pasteur-type effect in which glucose utilization is inversely related to oxygen concentration is observed in vascularly-perfused preparations (Lamers & Hülsmann, 1972; Hanson & Parsons, 1976; Porteous, 1978).

The specific activities of enzymes in rat small intestine show marked adaptive responses. In starvation, the changes parallel those seen in liver in that the specific activities of certain glycolytic enzymes fall, whereas those of certain gluconeogenic enzymes rise (Srivastava & Hübscher, 1966; Srivastava *et al.*, 1968; Stifel *et al.*, 1968; Shakespeare *et al.*, 1969; Stifel *et al.*, 1969; Anderson & Zakim, 1970; Mayer *et al.*, 1970; Weiser *et al.*, 1971; Shakespeare *et al.*, 1972; Anderson & Tyrrell, 1973; Anderson, 1974; Espinoza *et al.*, 1975, 1976; Crouzoulon, 1979).

In particular, the activities of hexokinase and phosphofructokinase in jejunal mucosa fall by about 47 and 43% respectively on starving rats for 36–72 h (Shakespeare *et al.*, 1972; Anderson, 1974). Although phosphofructokinase is found only in the soluble fraction of cell homogenates, hexokinase is found in both soluble and particulate fractions. Hexokinase activity in both fractions is decreased after starvation and the ratio of activity in the particulate to that in the soluble fraction is increased (Srivastava *et al.*, 1968). Hübscher and co-workers observed that the rate of glycolysis was decreased by starvation only when glucose, but not glucose 6-phosphate, was the substrate; the decrease was therefore ascribed to changes in the activity of hexokinase (Srivastava & Hübscher, 1966; Shakespeare *et al.*, 1969). Accordingly there have been many investigations of the adaptive response of hexokinase and its distribution between particulate and soluble fractions (Katzen *et al.*, 1970; Mayer *et al.*, 1970; Mayer & Hübscher, 1971; Weiser *et al.*, 1971; Jones & Mayer, 1972, 1973; Anderson & Tyrrell, 1973).

In comparison with hexokinase, the adaptive response of phosphofructokinase in intestinal mucosa has been little studied; moreover, this type of investigation has been confined to measurements of enzyme activity under optimal conditions (Shakespeare *et al.*, 1972; Anderson, 1974; Crouzoulon, 1979). However, mucosal phosphofructokinase is a complex, allosteric, enzyme (Tejwani & Ramaiah, 1971), so that adaptive changes in regulatory properties manifested under suboptimal conditions should also be of interest. For example, it has been reported that phosphofructokinase in crude extracts of rat liver shows an increased susceptibility to inhibition by ATP after rats have been starved, and this has been shown to be caused by a glucagon-mediated decrease in the concentration of a novel allosteric effector, fructose 2,6-bisphosphate (Nieto & Castano, 1980; Van Shaftingen *et al.*, 1980*a*,*b*; Furaya & Uyeda, 1980; El-Maghrabi *et al.*, 1981). In the present paper we describe changes in the regulatory properties of phosphofructokinase from intestinal mucosa that occur in rats on starvation.

Materials and methods

Male Wistar rats weighing between 220 and 260 g were either maintained on a standard laboratory diet (Oxoid, modified 41B) fed *ad libitum* or starved for 48 h with free access to water. Extracts of intestinal mucosa were prepared as follows. Rats were anaesthetized with Sagatal (0.1 ml/100 g body wt.) and the abdominal cavity of each was incised. The small intestine was removed and divided into five sections of equal length (from the Ligament of Treitz to the ileocaecal valve), arbitrarily designated jejunum 1 and 2 (J1 and J2) and ileum 1, 2 and 3 (I1, I2 and I3). The lumen of each section was flushed with ice-cold homogenization buffer, slit longitudinally and blotted gently with a tissue to remove surplus fluid. The mucosa was then collected by scraping the surface of the lumen with a microscope slide and immediately frozen in liquid N_2. The frozen mucosa was weighed and homogenized directly, without being thawed, in a Potter–Elvehjem homogenizer with 5 vol. (v/w) of extraction buffer, composed of 50 mM-Tris/HCl buffer, pH 8, containing 100 mM-$(NH_4)_2SO_4$, 30 mM-KF, 1 mM-2-mercaptoethanol and, as proteinase inhibitors, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-6-amino-n-hexanoic acid and 0.5 mg of soya-bean trypsin inhibitor/ml. The homogenate was centrifuged at 75000 ***g*** for 30 min at 4°C, the pellet was discarded and the particle-free supernatant was stored on ice. Under these conditions there was no significant proteolysis of phosphofructokinase, as determined by electrophoresis up to 3 h after homogenization (S. M. Khoja & G. L. Kellett, unpublished work), nor was there any observable change in the regulatory properties of phosphofructokinase. Rapid and extensive proteolysis occurs in the absence of proteinase inhibitors. The optimal activity of the enzyme was determined at pH 8 and 27°C (Ling *et al.*, 1965), and the regulatory properties of the

enzyme were determined at pH 7 as described previously (Hussey *et al.*, 1977), except where specified. One unit of activity is defined as the formation of 1 μmol of fructose 1,6-bisphosphate/min.

Biochemicals were obtained from Sigma Chemical Co. or Boehringer, and other reagents were of analytical grade.

Results

Early studies on the rate of glycolysis in rat small intestine were performed by incubation of particle-free supernatants from mucosal homogenates in a medium containing 40mM-potassium phosphate buffer, pH 7.8 (Srivastava & Hübscher, 1966). However, when the fructose 6-phosphate saturation curve was determined under these conditions for phosphofructokinase in a crude jejunal extract of mucosa, it was found to be hyperbolic, and the curve for a preparation from a fed rat was indistinguishable from that for a preparation from a rat starved for 48h (Fig. 1). The regulatory properties of the enzyme were therefore subsequently determined at pH 7.0 as described previously for rabbit muscle phosphofructokinase (Hussey *et al.*, 1977). This assay medium contains 10mM-$(NH_4)_2SO_4$. The latter is a true activator of the enzyme rather than a de-inhibitor (Abrahams & Younathan, 1971), so that, although a concentration of 10mM is more than 10-fold that required to saturate phosphofructokinase at pH 7, the enzyme still displays regulatory properties under these conditions. The extraction buffer contained 100mM-$(NH_4)_2SO_4$ in order to stabilize the enzyme (Ho & Anderson, 1971), but any $(NH_4)_2SO_4$ carried over by the portions of extract (5–15 μl) added into the assay mixture did not affect the regulatory properties of phosphofructokinase because $(NH_4)_2SO_4$ was already saturating.

The distribution of total phosphofructokinase activity (pH 8 assay) in the mucosa of the small intestines of fed and 48h-starved rats is shown in Fig. 2. Two features are prominent: first, phosphofructokinase displays a negative aboral gradient of activity from jejunum to ileum; secondly, the gradient is decreased on starvation, though by no means abolished, entirely because of a decrease in the specific activity of the enzyme in jejunal sections. Table 1 gives the relevant data for sections J1 and I3 expressed in units/mg of protein rather than units/g wet wt. of mucosa.

The regulatory properties of phosphofructokinase from rat intestinal mucosa are also affected by starvation. For the purpose of studying this effect, the activity observed at pH 7.0, v, is expressed as a ratio to that under optimal conditions at pH 8, V, by the quantity v/V, so that the effect on regulatory properties may be separated from that on enzyme specific activities. Fig. 3(*a*) shows that, after rats have been deprived of food, the fructose

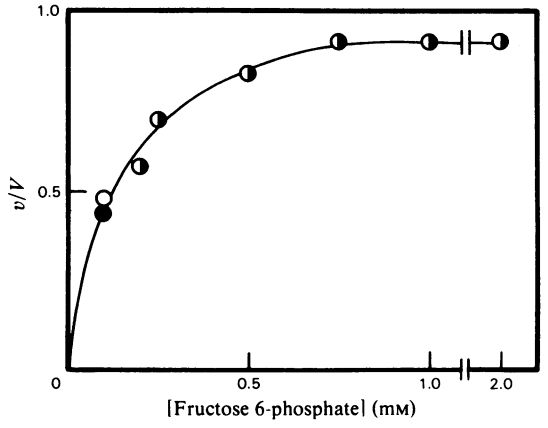

Fig. 1. *Dependence of mucosal phosphofructokinase activity on fructose 6-phosphate concentration under conditions equivalent to those used by Srivastava & Hübscher (1966) for measurements of glycolytic rate*

Phosphofructokinase activity was assayed in the particle-free supernatant of a mucosal extract from the jejunum of a fed rat (○) and a 48h-starved rat (●) (overlapping points are indicated by ◑). The assay conditions were those described by Ling *et al.* (1965), except that the pH was 7.8 and the cuvette also contained K_2HPO_4 (40mM). For further experimental details see the text.

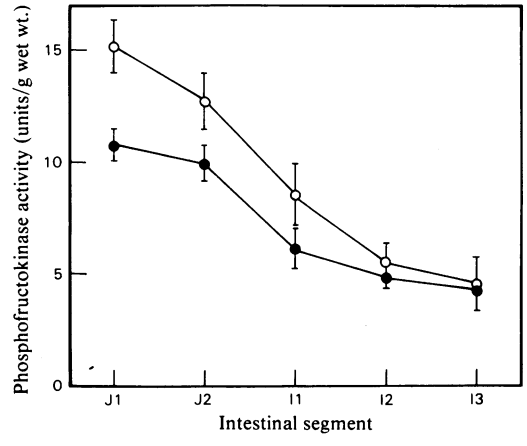

Fig. 2. *Effect of starving rats on regional specific activity of phosphofructokinase in the mucosa of the small intestine*

The optimal assay conditions described by Ling *et al.* (1965) were used. Activities were measured in preparations from fed rats (○) and from rats starved for 48h (●). For further experimental details see the text. Values shown are means ± S.D. for five rats. P values: J1, <0.001; J2, <0.005; I1, <0.005; I2 and I3, not significant.

Table 1. *Specific activities and activity ratios of mucosal phosphofructokinase in the proximal jejunum and terminal ileum of rat small intestine*

For full experimental details see the text. For the starved and re-fed rats, the starved rats were re-fed *ad libitum* on standard laboratory diet and water containing glucose (10%, w/v) overnight for 16h. Results are given as means ± S.D., with the numbers of rats given in parentheses. *P* values are given for comparison with corresponding data for fed rats (N.S., not significant).

	J1 segment				I3 segment			
Status of rat	Specific activity (nmol/min per mg of protein)		$v_{0.5}/V$		Specific activity (nmol/min per mg of protein)		$v_{0.5}/V$	
Fed	184 ± 15 (5)		0.45 ± 0.08 (23)		72 ± 18 (5)		0.41 ± 0.07 (6)	
Starved	122 ± 8 (5)	$P<0.01$	0.21 ± 0.03 (14)	$P<0.01$	67 ± 14 (6)	N.S.	0.20 ± 0.07 (5)	$P<0.01$
Starved and re-fed	170 ± 5 (4)	N.S.	0.41 ± 0.12 (4)	N.S.	74 ± 11 (4)	N.S.	0.41 ± 0.06 (4)	N.S.

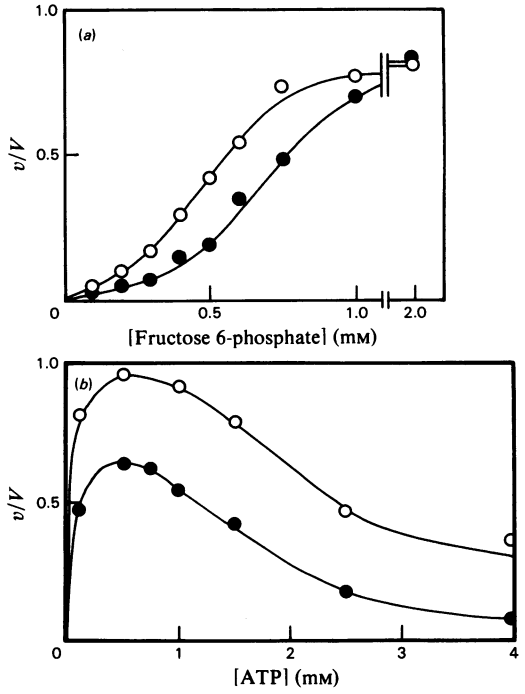

Fig. 3. *Effect of starving rats on the regulatory properties of phosphofructokinase in the jejunal mucosa of the small intestine*

Enzyme activity was assayed at pH 7 as described in the Materials and methods section, with crude (particle-free) extracts derived from the jejunal mucosa from fed rats (○) and from rats starved for 48h (●). For full experimental details see the text. (*a*) Dependence of activity on fructose 6-phosphate concentration in the presence of ATP (2.5mM); (*b*) dependence of activity on ATP concentration in the presence of fructose 6-phosphate (0.5mM).

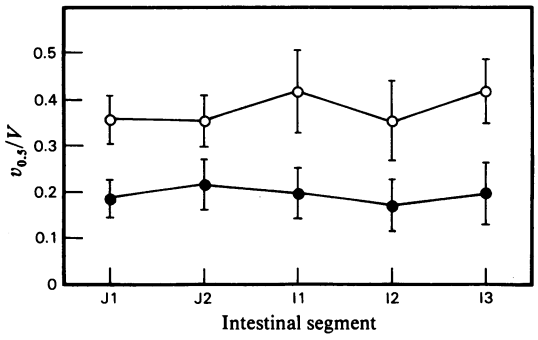

Fig. 4. *Effect of starving rats on the activity ratio of mucosal phosphofructokinase in different regions of the small intestine*

Assay conditions and symbols were as indicated in Fig. 3 legend. The results were obtained from the same five rats as used for the determination of specific activities in Fig. 2, and the values shown are means ± S.D. *P* values: J1, <0.001; J2, <0.005; I1, <0.002; I2, <0.005; I3, <0.001.

6-phosphate saturation curve of jejunal phosphofructokinase in the presence of 2.5mM-ATP is shifted to higher concentrations, from an apparent K_m in fed rats of 0.47mM to one of 0.66mM in starved rats. This increase in K_m for fructose 6-phosphate is a reflection of increased susceptibility to inhibition by ATP after starvation (Fig. 3*b*). At 0.5mM-fructose 6-phosphate, the difference in activity of phosphofructokinase between extracts from fed and starved rats is almost maximal, and the overall rates, even with ileal samples, are fast enough for convenient measurement. The influence of starvation on the regulatory properties of phosphofructokinase from different sections of the small intestine was therefore studied at 0.5mM-fructose 6-phosphate and 2.5mM-ATP in multiple samples. The data in Fig. 4 show that there is no difference in susceptibility to inhibition by ATP between jejunum and ileum either in a fed or in a starved rat, and that the difference in susceptibility between the jejunum of a fed and the jejunum of a starved

rat is maintained along the length of the intestine to the ileum.

In determining the regulatory properties of phosphofructokinase we were concerned that the delay of 1–2 min incurred between excision of the intestine and homogenization of the mucosal scrape might cause a change in regulatory properties. Several experiments were therefore performed in which a segment of intestine was washed with ice-cold saline (0.9% NaCl) *in situ* in an anaesthetized rat and mucosa was then extracted with the device described by Bronk & Leese (1973). In this way, mucosal samples could be homogenized within 10 s of removal. No differences in the regulatory properties of these samples and the usual scrape sample were observed.

It is difficult to be precise in the quantitative assay of the regulatory properties of phosphofructokinase, for even minor differences in the assay procedure, such as small changes in concentrations of substrates, in the steady-state concentrations of products or in pH, cause day-to-day variations in experimental results obtained (Emerk & Frieden, 1975). For this reason, the comparisons between fed and starved rats in Figs. 2 and 4 were performed in groups of two fed and two starved rats on the same day for three separate days. The equivalent data in Table 1 were collected from larger numbers of rats over a longer period.

When previously starved rats were re-fed overnight for 16 h on a high-carbohydrate diet, the specific activities and the regulatory properties of phosphofructokinase were restored to normal values (Table 1).

Discussion

The conclusion that hexokinase is the rate-limiting enzyme of glycolysis in intestinal mucosa when glucose is the substrate rests almost exclusively on measurements of the rate of lactate production from different substrates in particle-free supernatants prepared from the mucosa of whole intestine (Srivastava & Hübscher, 1966). These measurements were made by incubating a portion of crude extract with 4–5 vol. of an incubation medium containing glucose (10 mM) and buffered at pH 7.8 with phosphate (40 mM). The regulatory properties of phosphofructokinase in crude mucosal extracts had not been described at the time of the studies made by Srivastava & Hübscher (1966); unfortunately, it is now apparent from the data presented in Fig. 1 that, under those incubation conditions, phosphofructokinase is non-co-operative and its activity is unrestrained by any allosteric inhibitors present in the extracts from either fed or starved rats. Since the activity of phosphofructokinase in the jejunal mucosa of fed rats is 15.2 units/g wet wt. (Fig. 2 and Table 1; see also Shakespeare *et al.*, 1972), whereas that of hexokinase is only 2.7 units/g wet wt. (Shakespeare *et al.*, 1972), hexokinase would of necessity be rate-limiting in those circumstances. A similar conclusion is true also for starved rats. The design of the experiments performed by Srivastava & Hübscher (1966) therefore does not permit any decision as to whether hexokinase or phosphofructokinase is the rate-limiting enzyme of glycolysis in intestinal epithelial cells. Similar comments about the use of phosphate apply to the experiments performed by Clark & Sherratt (1967).

In the context of the question of rate-limiting enzymes, it is noteworthy that the rate of glycolysis, as reflected either by the rate of glucose utilization or by the rate of lactate production, in the ileum is only about 50–60% that of the jejunum (Hanson & Parsons, 1976). However, the total activity of hexokinase is actually 30–100% higher in the ileum (Weiser *et al.*, 1971; Crouzoulon, 1979; see, however, Hanson & Carrington, 1981). Indeed, of the 13 enzymes of glucose and fructose metabolism studied by Crouzoulon (1979), only hexokinase showed a positive aboral gradient of activity; all the others showed negative aboral gradients, with activities in the terminal ileum ranging from 74% to 3.5% of that in the proximal jejunum (see also Anderson & Zakim, 1970; Nakayama & Weser, 1972; Espinoza *et al.*, 1975, 1976). In addition, vascular glucose utilization by the jejunum in the streptozotocin-diabetic rat is only about 70% of normal (Hanson & Parsons, 1978), whereas hexokinase activity is increased by 25% (Anderson, 1974). The suggestion that the hexokinase activity is rate-limiting (Srivastava & Hübscher, 1966) is not consistent with either observation. Hanson & Carrington (1981) have also cast doubt on the idea that hexokinase is rate-limiting in glycolysis in rat small intestine.

Phosphofructokinase activity in the mucosa of fed rats decreases aborally along the length of the small intestine so that the activity in the terminal ileum (I3) is about 30% of that in the proximal jejunum (J1) (Fig. 2). This value is in excellent agreement with that of 32% reported by Crouzoulon (1979). When rats were starved for 48 h, total phosphofructokinase activity in jejunal mucosa decreased by about 30% ($P < 0.001$; Fig. 2). This value is somewhat smaller than that of 43–46% found by Shakespeare *et al.* (1972) and Anderson (1974). With the exception of hexokinase (Weiser *et al.*, 1971), there does not appear to have been any study on the effect of starvation on enzyme specific activities in the ileum; hence the effect of starvation on the gradient of activity has not been established for any glycolytic enzyme showing the normal, negative, aboral gradient.

Fig. 2 shows that starvation has no significant

effect on the phosphofructokinase specific activity in the ileal sections I2 and I3. Thus the result of starvation is a diminution in the gradient of phosphofructokinase activity caused largely by a decrease in the activity of the jejunal segments (J1 and J2). Overnight re-feeding of starved rats on a high-carbohydrate diet restores the phosphofructokinase gradient to its original state in fed rats (Table 1). Previous studies in which rats have been maintained on selected diets suggest that the aboral gradient of glycolytic enzyme activity is caused not by the failure of dietary carbohydrate to reach the ileum, but rather is an intrinsic characteristic of the small intestine (Espinoza *et al.*, 1975, 1976; Crouzoulon, 1979). The present data strongly support the latter idea, for during starvation no region of the lumen contains dietary carbohydrate and yet the aboral gradient of phosphofructokinase activity is not abolished. It is to be remembered, of course, that the small intestine is a unique tissue, in that glucose is supplied by two distinct routes, from the lumen and from the vasculature, and that the latter route still operates during starvation. The importance of the vascular route is emphasized by the finding by Espinoza *et al.* (1976) that perfusion of the ileum with glucose induces increases in the activities of glycolytic enzymes in the jejunum and vice versa, presumably mediated by a humoral mechanism.

The starvation of rats for 48h results in an increased susceptibility of mucosal phosphofructokinase to inhibition by ATP, the value of the activity ratio ($v_{0.5}/V$) in the segment J1 changing from 0.36 ± 0.05 to 0.19 ± 0.04 ($P < 0.001$) as a result of an increase in apparent K_m for fructose 6-phosphate from 0.47 to 0.66mM on starvation of the animals (Fig. 3). In fed rats, the susceptibility of phosphofructokinase to inhibition by ATP is independent of location in the small intestine, there being no significant difference in the activity ratio between any of the segments J1 to I3. The same is true for starved rats, so that the difference in susceptibility of phosphofructokinase to inhibition by ATP between fed and starved rats is maintained along the length of the small intestine. Thus, with respect to the determination of the regulatory properties of phosphofructokinase, the response of the ileum to starvation is the same as that of the jejunum. This is in contrast with the adaptive changes in enzyme specific activities, where a decrease occurs in the jejunum, but not in the ileum (Fig. 2). Moreover, in the fed rat, for example, the specific activity of phosphofructokinase decreases from 15.2 ± 1.0 units/g wet wt. in segment J1 to 4.6 ± 1.2 units/g wet wt. in segment I3, though the activity ratio does not change. Hence the mechanism that determines changes in regulatory properties is distinct from that controlling enzyme specific activities. Since, in the fed rat, dietary carbohydrate is thought not to reach the terminal ileum (I3), the fact that the activity ratio (0.42 ± 0.03) is not the same as that in a starved rat (0.20 ± 0.07) emphasizes once more the importance of humoral factors in determining the properties of glycolytic enzymes.

Hanson & Parsons (1978) have reported that the utilization of glucose by the jejunum is decreased to 36% of that in fed rats when rats are starved for 48h. Since the utilization of glucose by the smooth muscle is very much less than that of the mucosa (Leese & Bronk, 1975), the change in utilization primarily reflects changes in the glucose metabolism of mucosa. Changes in specific activities of glycolytic enzymes are not sufficient to account for the decrease in glucose utilization, since even the hexokinase specific activity is decreased only to 55% of its value for fed rats in starved rats (Shakespeare *et al.*, 1972). However, if the existence of a Pasteur effect in vascularly-perfused jejunum (Hanson & Parsons, 1976) is taken as evidence that phosphofructokinase is the principal rate-limiting enzyme of glycolysis, then the decrease in glucose utilization rate can be accounted for by a combination of changes in the specific activity and in the regulatory properties of the enzyme. For example, the data of Figs. 2 and 4 show that the specific activity of phosphofructokinase would be decreased to 37% of the value for fed rats on starvation. This excellent agreement with the glucose utilization rate in starved rats is, of course, fortuitous, since it depends on an arbitrary choice of suboptimal assay conditions. However, the calculation emphasizes that changes in the specific activity and regulatory properties of phosphofructokinase in jejunal mucosa can in principle account for changes in glucose utilization on starvation.

The increase in susceptibility of phosphofructokinase activity to inhibition by ATP when assayed in crude mucosal extracts from starved rats is similar to that observed for liver phosphofructokinase (Nieto & Castano, 1980). It remains to be established whether mucosal phosphofructokinase activity is regulated by fructose 2,6-bisphosphate (Van Shaftingen *et al.*, 1980*a*,*b*).

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